this month in

GASTROENTEROLOGY *(continued)*

a follow-up study of ferritin synthesis in GH using in situ hybridization and immunohistochemical techniques on duodenal biopsy specimens from patients with GH, patients with nongenetic iron overload, and controls. Ferritin messenger RNA levels were reduced in GH biopsy specimens, as shown earlier, and the ferritin messenger RNA signal was low in both absorptive and nonabsorptive cells. Another important finding reported here was that the levels of iron regulatory factor, a protein that inhibits the expression of ferritin, was higher in patients with GH than controls. These results suggest that low duodenal ferritin levels in GH result from low expression of ferritin messenger RNA and high levels of iron-regulatory factor. The next step is to unravel the cause of these findings. . . . *page 208*

Heartburn by the Can

The pause that refreshes may be directly related to heartburn symptoms, according to a interesting study by Feldman and Barnett from Dallas. The authors measured the pH, titratable acidity, and osmolality of 38 popular beverages and correlated these in vitro findings with the ability of each drink to cause heartburn as assessed by a questionnaire given to 394 people with heartburn. The authors present a large table with the osmolality, pH, titratable acidity, and heartburn score for all 38 drinks. Journal readers who have heartburn may have a personal as well as professional interest in these fascinating results. The major offenders were grapefruit juice, orange juice, red wine, and coffee. Frequent reflux symptoms were associated with high titratable acidity of citrus drinks and juices and with low pH of soft drinks. These results will be useful in managing patients with heartburn who request detailed dietary recommendations. . . . *page 125*

New Crohn's Clues

First-degree relatives of patients with Crohn's disease are at increased risk of eventually developing the disease. Some of these apparently healthy relatives also show increased intestinal permeability, a condition commonly observed in the majority of patients with Crohn's disease. The cause of increased intestinal permeability in patients and their relatives is not known, but Yachshyn and Meddings from Calgary describe an immune mechanism that may be responsible. The authors recently reported that patients with Crohn's disease but not patients with either ulcerative colitis or celiac disease express a B cell marker, CD45RO, that is also expressed on memory T cells. Because expression of CD45RO follows antigenic stimulation, the authors postulated that relatives of patients with Crohn's disease with increased intestinal permeability and increased antigen exposure would also express CD45RO on their peripheral B cells. They studied intestinal permeability and CD45RO expression in 15 patients with Crohn's disease and 13 of their first-degree relatives. A subset of first-degree relatives had elevated intestinal permeability and an increase in the percentage of B cells expressing the CD45RO marker. This intriguing observation may provide an important clue for eventually solving the Crohn's puzzle. . . . *page 132*

Cholera Toxin: The Big Picture

Recent evidence suggests that the well-known local effects of cholera toxin on enterocyte cyclic adenosine monophosphate do not entirely explain how this toxin causes diarrhea. It is now recognized that the enteric nervous system and enteroendocrine cells work together to amplify the signal from the lumen to cause watery diarrhea. In this issue, Nocerino et al. from Italy provide new insight into the neuronal mechanisms of cholera diarrhea. The investigators used the rat ileal loop model to determine if cholera toxin in the jejunum had a remote secretory effect on colonic water and electrolyte secretion. When the jejunum was exposed to cholera toxin but not other secretagogues, the researchers observed water and electrolyte secretion in the colon. But when the small intestine was transected below the jejunum, cholera toxin in the jejunum had no effect on colonic secretion. These interesting results suggest that cholera toxin sends a secretory signal to distant parts of the intestine via the enteric nervous system. These experiments also are relevant to previous reports of impaired colonic absorption in patients with secretory diarrhea, including cholera. . . . *page 34*

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ALIMENTARY TRACT

Galanin Contracts and Relaxes Guinea Pig and Canine Intestinal Smooth Muscle Cells Through Distinct Receptors

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Background/Aims: Galanin induces a contraction or a relaxation of digestive smooth muscle. Receptors mediating these effects have not been pharmacologically characterized. The aim of the study was to evaluate properties of two specific galanin antagonists M15 and M35 on galanin effects on muscle cells. *Methods:* Isolated muscle cells were obtained separately from circular and longitudinal layers of guinea pig and dog ileums. Contraction was expressed as percentage decrease in cell length from control. *Results:* Galanin induced a contraction of cells from guinea pig circular layer (50% effective concentration [EC_{50}], 80 pmol/L) and dog longitudinal layer (EC_{50}, 100 pmol/L). The antagonists inhibited galanin-induced contraction. The most potent was M15 (50% inhibitory concentration [IC_{50}], 80 pmol/L in guinea pig; 90 pmol/L in dog) which was >M35 (IC_{50}, 4 nmol/L in guinea pig; 1 nmol/L in dog). In dog circular layer, galanin inhibited cholecystokinin-induced contraction by relaxing the cells (EC_{50}, 3 pmol/L). The antagonists inhibited this relaxation. The most potent was M35 (IC_{50}, 60 pmol/L) which was >M15 (IC_{50}, 900 pmol/L). *Conclusions:* Galanin antagonists M15 and M35 inhibit the contraction and the relaxation induced by galanin with different potency, suggesting the presence of distinct galanin receptors in gastrointestinal tract that each mediates a specific effect.

In the gastrointestinal tract, galanin has been found in the neural elements of submucosa and muscle layers and in the pancreas.[1,2,3] Galanin regulates the activity of digestive smooth muscle in several mammalian species, in which it shows species-specific stimulatory[1,4–6] or inhibitory effects.[7–10] These actions may result from either a direct myogenic effect[11–14] or occur indirectly via the release of other neurotransmitters.[9,14–16] We have shown previously that galanin induces a contraction of isolated cells from the circular muscle layer of pig, guinea pig, rat, and rabbit ileum, whereas it induces a cell relaxation in dog ileum circular muscle layer.[17,18] These results are in agreement with in vivo studies described above, suggesting a physiological role for the myogenic action of galanin in these species and the presence of different galanin receptor types on smooth muscle. However, these receptors have not been characterized until now.

Based on the observation that an intact N-terminal sequence is needed for a full action of galanin, a series of chimeric peptides have been synthesized using the 1-13 N-terminal fragment of galanin and C-terminal portions of some other bioactive peptides. Several galanin receptor antagonists were observed among these chimeric peptides, most of which also possess higher affinity for the galanin receptor than galanin itself.[19] These peptides antagonize the actions of galanin in each galanin-receptive system tested so far. Notably, the galanin-mediated inhibition of insulin release from pancreatic islets was fully reversed by M15 (also named galantide).[20] M15 blocks the facilitatory effects of galanin on the spinal flexor reflex.[21] Another chimeric peptide M35, which includes in its C-terminal portion a bradykinin 2-9 fragment, binds with high affinity to galanin binding sites in the central nervous system and shows an antagonistic effect at the receptor of galanin.[22]

Consequently, the present study was designed to (1) determine the ability of the two galanin antagonists M15 and M35 to inhibit the contraction induced by galanin on isolated smooth muscle cells from guinea pig ileum circular layer and dog ileum longitudinal layer and the relaxation induced by galanin in dog ileum circular layer; and (2) characterize pharmacologically the galanin receptor type respectively involved in these opposite effects of galanin on digestive smooth muscle. To assess the specificity of the action of galanin and galanin-antagonists on these cells, we compared their effects with those

Abbreviations used in this paper: CCK-8, cholecystokinin octapeptide; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration.

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of a widely evaluated contracting agent, cholecystokinin octapeptide (CCK-8).[23,24]

Materials and Methods

Materials

Collagenase (types I and V) and pronase were purchased from Boehringer Mannheim Ltd. (Meylan, France). Penicillin G and streptomycin G were purchased from Specia (Paris, France). Galanin, CCK-8, and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Galanin antagonists M15 [galanin-(1-12)-pro-substance P-(5-11) amide] and M35 [galanin-(1-13)-bradykinin-(2-9) amide] were obtained from Dr. Ülo Langel (Stockholm University, Stockholm, Sweden).

Cell Dispersion

Cell dispersion was achieved as previously reported.[17,25] Briefly, smooth muscle cells were isolated separately from the circular and longitudinal muscle layer of ileum from 1-year-old male beagle dogs and albino male guinea pigs (250–300 g body wt). Small muscle strips were incubated for two successive periods of 30 minutes at 31°C in a medium (132 mmol/L NaCl, 5.4 mmol/L KCl, 5 mmol/L Na_2HPO_4, 1 mmol/L NaH_2PO_4, 1.2 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 25 mmol/L HEPES, 0.2% glucose [wt/vol], 0.2% bovine serum albumin [wt/vol]; pH 7.4) bubbled with 95% O_2 and 5% CO_2 and supplemented with antibiotics, 100 IU/mL penicillin G, 50 μg/mL streptomycin, and 0.2 mg/mL soybean trypsin inhibitor). Enzyme concentrations were adapted according to the species: 0.2 mg/mL collagenase type I and 0.2 mg/mL pronase in dog and 0.1 mg/mL collagenase type I, 0.1 mg/mL collagenase type V, and 0.2 mg/mL pronase in guinea pig. At the end of each incubation, the medium was filtered, and the partly digested muscle strips were washed four times with enzyme-free medium. These strips were then transferred into fresh, enzyme-free medium and left to stand for 20 minutes to allow the muscle cells to disperse spontaneously under very slow mechanical agitation. Cells were harvested through a 500-μm nylon filter. It is emphasized that only those cells that had dissociated spontaneously in enzyme-free medium were used for functional measurements. Viability tests (exclusion of trypan blue) showed that 90% of cells in suspension were viable at the time of contraction experiments.

Measurement of Contractile Response

Cell suspensions were usually studied within 30 minutes of dispersion. Cell density of the suspension was about 250,000 cells/mL. Aliquots of 250 μL of cell suspension were added to 250 μL of the solution containing the agent to be tested, thereby ensuring rapid mixing, and were incubated for 30 seconds at 31°C. The reaction was interrupted by the addition of glutaraldehyde to a final concentration of 2.5%.

In control experiments, 250 μL of the same medium were substituted for the tested agent. To measure cell length, an aliquot of cells fixed with glutaraldehyde was placed on a Malassez slide (Paul Block, Strasbourg, France), and the length of the first 50 cells randomly encountered in successive microscopic fields was measured. Only cells that were whole at microscopic examination were measured. Cell length measures were performed on a video screen; a video camera recorded the microscopic image, and a scale mask was placed on the screen. Magnification caused by the video recording had first been calculated by comparison with length measures obtained with the image splitting eye piece connected to a micrometer.

Experiments of Inhibition or Relaxation

For relaxation experiments, cells were preincubated for 1 minute in the presence of various concentrations of the inhibitory or relaxing agents to be tested. Then the contracting agent was added, and the reaction was stopped after 30 seconds, as described above. In preliminary experiments, we tested the influence of different schedules of peptide administration. We compared the "relaxing" effect of galanin and vasoactive intestinal polypeptide (VIP) when galanin or VIP were added to the medium before, at the same time, or after CCK, and we did not observe any difference in their "relaxing" effect.

Expression of Results

The contractile response was defined as the decrease in the average cell length of a population of muscle cells exposed to a tested agent in comparison to controls. Cell contraction was expressed as the percentage of decrease in cell length from control. The decrease in cell length was calculated using the following formula: $([L_0 - L_x]/L_0) \times 100$. L_0 is the mean length of cells in resting state, and L_x is the mean length of treated cells.

In relaxation experiments, the degree of inhibition was expressed as the percentage decrease in the contractile response from the maximal response observed in the absence of inhibitors, taken as 100%. Throughout this paper, n refers to the number of experiments; each was performed on samples from different animals. Statistical evaluation was performed using the Student's *t* test, and the normality of the cell samples was assessed by the normal law test of Kolmogorov–Smirnov.

Calculation of Schild Plots

Isolated ileal smooth muscle cells were preincubated for 1 minute in the presence of definite concentrations of galanin antagonists (M15 or M35), ranging between 0.01 and 100 nmol/L. Then, increasing concentrations of galanin were added to determine the concentration-response curve to galanin. After 30 seconds, the reaction was stopped by the addition of glutaraldehyde. For each concentration of M15 or M35, 50% effective concentration (EC_{50}) of galanin was determined. In the representation $y = f(x)$ in which $y = \log (x - 1)$ with $x = EC_{50}$ of galanin with a fixed concentration of antagonist (M15 or M35) divided by EC_{50} of galanin without antagonist and in which the concentration of galanin antagonist is reported on the *x* axis, the intersection point between the *x* axis and the straight line joining the four EC_{50} indicated the pA2 value.

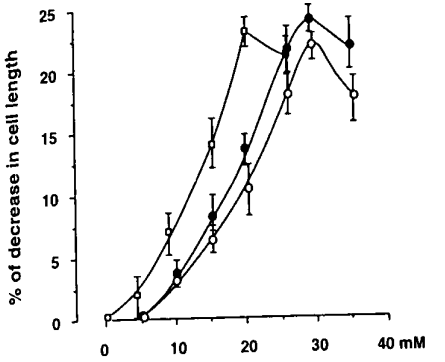

Figure 1. Contracting effect of increasing concentrations of KCl in ileal smooth muscle cells from guinea pig circular layer (□) and dog circular (○) and longitudinal (●) layer. Cells were incubated for 30 seconds at 31°C with increasing concentrations of KCl and then fixed by 2.5% glutaraldehyde. Results are expressed as the percentage of cell length decrease from control. Values are means ± SEM of four separate experiments.

Results

Length of Resting Isolated Cells and Response to Increasing Concentrations of KCl

Length of isolated cells was measured after an incubation of 30 seconds in the sole presence of the medium and fixation by glutaraldehyde at a final concentration of 2.5%. Mean length of cells isolated from the circular layer of guinea pig ileum was 91 ± 16 μm (mean ± SEM of the length of 350 cells). In the dog, the mean length was 112 ± 16 μm for cells obtained from the circular layer of the ileum and 137 ± 18 μm for cells from the longitudinal one (mean ± SEM of the length of 350 cells).

When cells were incubated in the presence of increasing concentrations of KCl, ranging from 5 to 40 mmol/L, cells were contracted in a concentration-dependent manner. Maximal contraction was obtained at 20 mmol/L of KCl for cells from the guinea pig's ileum circular layer and at 30 mmol/L of KCl for cells from the dog's circular and longitudinal layer (mean of four experiments from separate animals) (Figure 1).

Effects of Galanin and CCK-8 in Dog and Guinea Pig Ileum

Circular layer from guinea pig ileum and longitudinal layer from dog ileum. As previously demonstrated,[17] galanin and CCK-8 induced a contraction of isolated smooth muscle cells of the circular layer from guinea pig ileum and of the longitudinal layer from dog ileum in a concentration-dependent manner. The effect of both galanin and CCK was not inhibited when cells were incubated in the presence of 10 μmol/L of tetrodotoxin (data not shown). The maximal contraction induced by galanin was obtained at 10 nmol/L in both species and corresponded to a 25.7% ± 3.5% and 24.2% ± 2.2% decrease in cell length from control in guinea pig and dog, respectively. The concentration inducing a half-maximal contraction (EC_{50}) was 80 pmol/L in guinea pig and 100 pmol/L in dog.

CCK-8 induced a maximal cell contraction at 1 nmol/L in guinea pig and at 10 nmol/L in dog, which corresponded to a 24.5% ± 2.3% and 24.5% ± 1.8% decrease in cell length from control in guinea pig and dog, respectively. EC_{50} was 9 pmol/L in guinea pig and 60 pmol/L in dog. Data concerning the effect of galanin and CCK are summarized in Table 1.

Circular layer from dog ileum. In the dog ileum circular layer, we have previously shown that CCK-8 induced a contraction of smooth muscle cells in a concentration-dependent manner.[17] The maximal contraction was observed at 1 nmol/L of CCK-8 and was 26.1% ± 3.1% of the resting cell length. The EC_{50} was 50 pmol/L.

When the cells were incubated with increasing concentrations of galanin alone, the length of the cells was unaffected. By contrast, galanin inhibited cell contraction induced by a maximal concentration of CCK-8 (10 nmol/L) in a concentration-dependent manner. The CCK-8–induced contraction was abolished at 10 nmol/L of galanin. EC_{50} of galanin was 3 pmol/L (Table 1).

Effect of M15 and M35 on Cell Contraction Induced by CCK-8 and Galanin

Specificity of galanin antagonists. In all cell types (circular layer from guinea pig and dog or longitudinal layer from dog), incubation of cells in the sole presence of either M15 or M35 did not alter cell length. M15 (1 μmol/L) and M35 (1 μmol/L) also did not alter the cell contraction induced by CCK-8 that was inhibited by L365,260 (1 μmol/L), a specific CCK antagonist (Figure 2, Table 2). Moreover, M15 and M35 did not alter the response to KCl of isolated cells from any of the layers studied (Table 2).

Effect of galanin antagonists on cell contraction induced by galanin. In circular layer from guinea pig and longitudinal layer from dog ileum, M15 and M35 inhibited the contraction induced by galanin (10 nmol/L) in a concentration-dependent manner. The potency of the antagonists was characterized by the concentration inhibiting the effect of galanin by 50% (50% inhibitory

Table 1. Comparison of Effect of Galanin and CCK-8 on Isolated Smooth Muscle Cells From Guinea Pig and Dog Ileum

	Guinea pig (circular layer)			Dog (longitudinal layer)			Dog (circular layer)		
	Effect	EC_{50} (pmol/L)	C_{max} (nmol/L)	Effect	EC_{50} (pmol/L)	C_{max} (nmol/L)	Effect	EC_{50} (pmol/L)	C_{max} (nmol/L)
Galanin	Contraction	80	10	Contraction	100	10	Relaxation	3	10
CCK-8	Contraction	9	1	Contraction	60	10	Contraction	50	1

EC_{50}, mean value of effective concentration for half-maximal effect calculated from the individual dose-response curves of five separate experiments; C_{max}, concentration inducing a maximal effect.

concentration [IC_{50}]), calculated from concentration-response curves where mean values of five separate experiments were plotted. Galanin-induced contraction was abolished at 1 μmol/L M15 and 100 μmol/L M35 in both species. The most potent antagonist was M15 (IC_{50}, 80 pmol/L in guinea pig and 90 pmol/L in dog) > M35 (IC_{50}, 4 nmol/L in guinea pig and 1 nmol/L in dog) (Figures 3 and 4, Table 3).

Effect of Galanin Antagonists on Cell Relaxation Induced by Galanin

To test the action of M15 and M35 on the relaxing effect of galanin on circular smooth muscle cells from dog ileum, cells were first incubated with increasing concentrations of these drugs for 1 minute. Galanin (10 nmol/L) was then added for 1 minute, and finally, 10 nmol/L of CCK-8 was added for 30 seconds, and the cells were fixed. At a concentration of 1 μmol/L, M15 and M35 ended the relaxing effect of galanin, whereas the contraction induced by 10 nmol/L of CCK-8 persisted (Figure 5). The inhibition of the relaxing effect of galanin by M15 and M35 was concentration dependent. The most potent antagonist was M35 (IC_{50}, 60 pmol/L) > M15 (IC_{50}, 900 pmol/L) (Figure 5, Table 3).

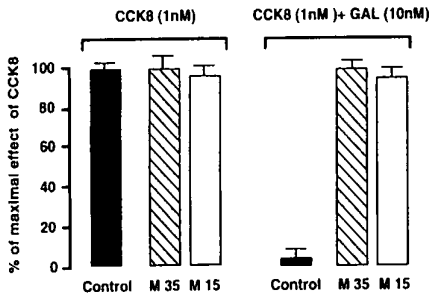

Figure 2. Specificity of action and inhibitory effects of specific antagonists of the galanin receptors M15 and M35 on CCK-8–induced contraction and galanin-induced relaxation in smooth muscle cells from circular layer of dog ileum. In contractile experiments, cells were incubated with 1 nmol/L CCK-8 alone (■) and with 1 nmol/L CCK8 + 1 μmol/L M15 (□) or 1 μmol/L M35 (■). In relaxing experiments, cells were incubated with 1 nmol/L CCK-8 + 10 nmol/L galanin (■) and with 1 nmol/L CCK-8 + 10 nmol/L galanin + 1 μmol/L M15 (□) or 1 μmol/L M35 (■). Results are expressed as the percentage of cell contraction observed in the absence of antagonists, taken as 100%. Points are means ± SEM of five separate experiments.

pA2 Values of M15 and M35

To further assess the pharmacological distinction between the two receptor types for galanin involved in functional studies of contraction and relaxation, we studied the influence of increasing concentrations of M15 and M35 on the effect of galanin on each of its receptors and calculated the pA2 values for both antagonists.

In guinea pig ileum circular smooth muscle cells, increasing concentrations of M15 (0.01, 0.1, 1, and 10 nmol/L) and M35 (0.1, 1, 10, and 100 nmol/L) caused a parallel rightward shift in concentration-response curves for galanin-induced contraction of the cells. A Schild plot of these data yielded a slope of 0.88 ± 0.06 for M15 (P = 0.10), showing that the slope was not statistically different from 1, and 0.91 ± 0.07 for M35 (P = 0.36), indicating a competitive antagonism of both antagonists at galanin receptors (mean ± SEM of five separate experiments). pA2 was 10.3 for M15 and 9.3 for M35, respectively. Likewise, increasing concentrations of M15 (0.01, 0.1, 1, and 10 nmol/L) and M35 (0.1, 1, 10, and 100 nmol/L) caused a right shift of concentration-response curve of galanin in dog ileum longitudinal smooth muscle cells. A Schild plot of these data yielded a slope of 0.77 ± 0.10 for M15 (P = 0.10) and 0.79 ± 0.10 for M35 (P = 0.10) (mean ± SEM of five separate experiments). pA2 was 10.4 for M15 and 9.1 for M35 (Figure 6, Table 4).

Similar experiments were performed on cells from the circular layer of dog ileum. Increasing concentrations of M15 (0.1, 1, 10, and 100 nmol/L) and M35 (0.01, 0.1, 1, and 10 nmol/L) shifted the concentration-response curves of the relaxing effect of galanin to the right. A Schild plot of these data yielded a slope of 0.88 ± 0.06 for M15 (P = 0.10) and 0.85 ± 0.08 (P = 0.36) for M35, indicating a competitive antagonism of both antagonists at galanin receptors. pA2 was 9.3 for M15 and 10.3 for M35 (Figure 6, Table 4).

Table 2. Specificity of Effect of M15 and M35 on Galanin-Induced Contraction on Isolated Smooth Muscle Cells From Guinea Pig and Dog

	% Contraction	
	Guinea pig (circular layer)	Dog (longitudinal layer)
CCK-8 (1 nmol/L)	24.5 ± 2.3	26.4 ± 2.8
Galanin (10 nmol/L)	25.7 ± 3.5	26.7 ± 3.2
KCl (20–30 mmol/L)	23.3 ± 2.4	24.1 ± 2.4
M15 (1 μmol/L) + CCK-8 (1 nmol/L)	24.1 ± 2.1	26.1 ± 3.0
M35 (100 μmol/L) + CCK-8 (1 nmol/L)	23.6 ± 2.5	25.2 ± 2.9
L365,260 (1 μmol/L) + CCK-8 (1 nmol/L)	2.1 ± 1.1	2.0 ± 1.2
M15 (1 μmol/L) + galanin (10 nmol/L)	2.8 ± 2.1	2.2 ± 2.5
M35 (100 μmol/L) + galanin (10 nmol/L)	2.2 ± 2.0	2.7 ± 1.4
L365,260 (1 μmol/L) + galanin (10 nmol/L)	25.1 ± 3.1	26.7 ± 3.6
M15 (1 μmol/L) + KCl (20–30 mmol/L)	22.8 ± 2.8	25.1 ± 2.8
M35 (100 μmol/L) + KCl (20–30 mmol/L)	23.1 ± 2.6	24.6 ± 2.4

NOTE. Contraction is expressed as the percent of cell length decrease from control (mean ± SEM, n = 5).

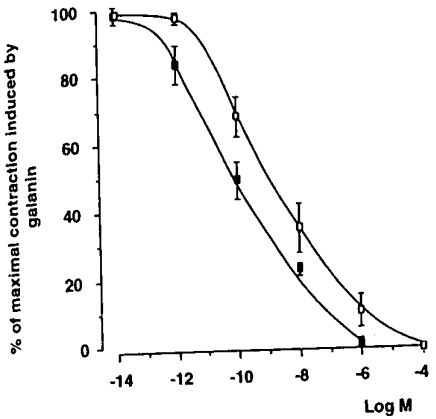

Figure 3. Inhibition by specific antagonists of the galanin receptors M15 and M35 of galanin-induced contraction in smooth muscle cells from longitudinal layer of dog ileum. Various concentrations of M15 (■) and M35 (□) were added to the medium for 1 minute. Cells were then stimulated by a maximal concentration of contracting agent (10 nmol/L galanin) for 30 seconds at 31°C. Results are expressed as the percentage of cell contraction observed in the absence of antagonists, taken as 100%. Points are means ± SEM of five separate experiments.

Discussion

These results confirm previous studies showing myogenic effects of galanin on ileal smooth muscle in dog and guinea pig. The use of specific antagonists allows the demonstration that these effects of galanin are mediated through its interaction with at least two different receptors, each receptor being responsible for a distinct effect: one mediates a relaxation and one mediates a contraction.

In the present study, we used the specific antagonists of galanin M15 and M35 for the first time on intestinal muscle cells. The action of M15 and M35 has been previously characterized on various cell types. These compounds bind with high affinity to galanin receptors.[19] Indeed, the galanin-mediated inhibition of insulin release from pancreatic islets was fully reversed by M15 with an IC_{50} of 1 nmol/L.[20] M15 also displaces ^{125}I-labeled galanin from its specific binding sites in membranes from the ventral hippocampus (IC_{50}, 100 pmol/L) and blocks the facilitatory effects of galanin on the spinal flexor reflex (IC_{50}, 100 pmol/L).[21] Morever, M15 acts as a reversible, high-affinity antagonist to block the inhibitory effects of galanin on the evoked release of acetylcholine in vivo in

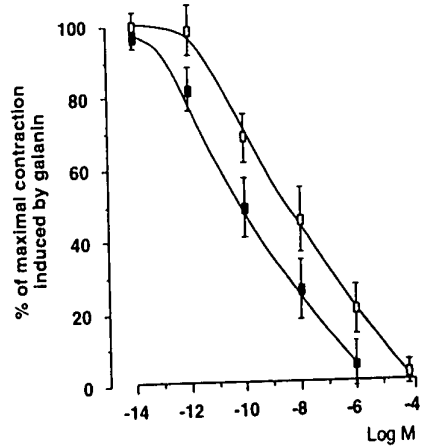

Figure 4. Inhibition by specific antagonists M15 and M35 of galanin induced contraction in smooth muscle cells from circular layer guinea pig ileum. Various concentrations of M15 (■) and M35 (□ were added to the medium for 1 minute. Cells were then stimulate by a maximal dose of contracting agent (1 nmol/L galanin) for 3 seconds at 31°C. Results are expressed as the percentage of ce contraction observed in the absence of antagonists, taken as 100% Points are means ± SEM of five separate experiments.

Table 3. Comparison of the Potency of Specific Galanin Receptor Antagonists M15 and M35 in Inhibiting the Effect Induced by Galanin in Isolated Smooth Muscle Cells From Guinea Pig and Dog Ileum

	Guinea pig (circular layer)		Dog (longitudinal layer)		Dog (circular layer)	
	IC_{50}	C_{max}	IC_{50}	C_{max}	IC_{50}	C_{max}
M15	80 pmol/L	1 μmol/L	90 pmol/L	1 μmol/L	900 pmol/L	1 μmol/L
M35	4 nmol/L	100 μmol/L	1 nmol/L	100 μmol/L	60 pmol/L	1 μmol/L

NOTE. Mean values were calculated from IC_{50} obtained from individual dose-response curves of five separate experiments.
C_{max}, concentration inducing a maximal inhibition; IC_{50}, concentration of antagonist inducing an inhibition of 50% of the maximal effect observed in the presence of antagonist.

the hippocampus[21] and on the galanin-induced hyperpolarization of locus caeruleus neurons in vitro.[21] Likewise, M35 displaces ^{125}I-labeled galanin from membranes of rat dorsal spinal cord with an IC_{50} of 300 pmol/L and dose-dependently antagonizes the effect of intrathecal galanin on the flexor reflex.[22]

On intestinal muscle cells, M15 and M35 specifically inhibited the effects of galanin. Indeed, M15 and M35 failed to inhibit the contraction induced by CCK-8 and KCl in both species. Moreover, when isolated smooth muscle cells from both species were incubated with concentrations of M15 or M35 ranging from 1 pmol/L to 1 μmol/L, no effect was observed. These results rule out a nonspecific inhibitory effect of these compounds and also eliminate a side-effect of M35 that contains a bradykininlike sequence because bradykinin is known to contract intestinal smooth muscle cells.[26]

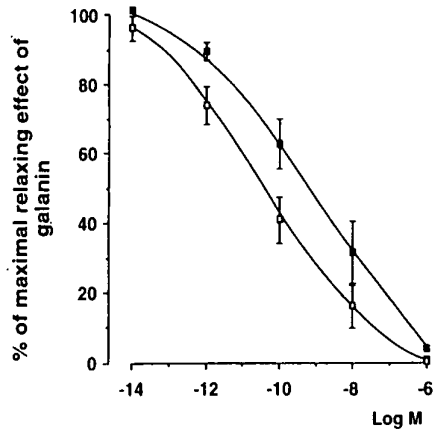

Figure 5. Inhibition by specific antagonists of the galanin receptors M15 and M35 of galanin-induced relaxation in smooth muscle cells from circular layer of dog ileum. Various concentrations of M15 (■) and M35 (□) were added to the medium for 1 minute. Galanin (10 nmol/L) was then added for 1 minute. Finally, 10 nmol/L of CCK-8 was added for 30 seconds, and the cells were fixed. Results are expressed as the percentage of cell contraction observed in the absence of antagonists, taken as 100%. Points are means ± SEM of five separate experiments.

When the Schild plots of M15 and M35 were calculated in the various types of smooth muscle cells we studied, we observed a negative value of log (x − 1) for the lowest dose of antagonist tested (10 pmol/L). This observation could suggest that these antagonists played a role of partial agonist or potentiated the effect of galanin when used at low concentrations. This seems not to be the case because we observed that the same concentration of antagonist showed a weak but significant inhibition of the response of cells to a maximal concentration of galanin and because the concentration-response curve to galanin in the presence of this low concentration of antagonist was very close to that of galanin in the absence of antagonist. Moreover, a partial agonist effect has not been shown for M15 until now. Such an effect has been shown for M35 in one study in which galanin was used to stimulate the striatal release of acetylcholine.[27] However, in the present study, M15 and M35 at concentrations ranging from 1 pmol/L to 1 μmol/L did not show any effect by themselves on isolated smooth muscle cells. They also did not show any agonistic effect when tested at the highest concentrations.

Many of the present results support the assumption that the receptor of galanin mediating its relaxing effect is pharmacologically distinct from the one mediating a contraction. When galanin induced a cell contraction (guinea pig circular muscle layer and dog longitudinal muscle layer), its EC_{50} was in the range of 100 pmol/L, whereas in dog circular muscle layer, when galanin induced a relaxation, its EC_{50} was 30-fold lower (3 pmol/L). Although EC_{50}s of agonists are not sufficient to discriminate receptors, they may indicate some differences in recognition of the receptors by agonist as observed, for example, in CCK receptors.[23]

Both antagonists inhibited the contraction and the relaxation induced by a maximal concentration of galanin in a concentration-dependent manner. However, comparing the relative potency of these antagonists, we observed that M15 was 20–60-fold more potent than M35 to inhibit the galanin-induced contraction in dog and guinea pig ileum. In contrast, M35 was 50-fold more potent than M15 to inhibit the galanin-induced relaxation in dog. These results strongly support the hypothesis that galanin-induced contraction and relaxation are mediated through two distinct receptors in intestinal smooth muscle.

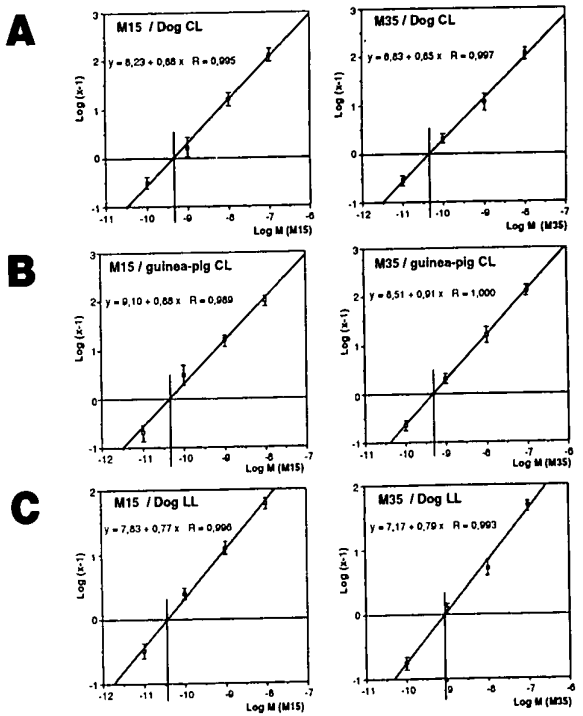

Figure 6. Schild plots of specific galanin receptor antagonists M15 and M35 in inhibiting the effect induced by galanin in isolated smooth muscle cells from (*A*) dog ileum circular (Dog CL), (*B*) guinea pig circular layer (guinea pig CL), and (*C*) dog longitudinal (Dog LL) layers (n = 6).

This hypothesis was further assessed by comparing the Schild plots and the pA2 values for M15 and M35 with the galanin receptor mediating a contraction and the receptor mediating a relaxation. These pA2 values clearly show that the effect of both antagonists was identical in

Table 4. Comparison of the pA2 Values of Specific Galanin Receptor Antagonists M15 and M35 in Inhibiting the Effect Induced by Galanin in Isolated Smooth Muscle Cells From Guinea Pig and Dog Ileum

Antagonists used	Species and muscle layers used	Slopes	pA2 values	
Galanin-M15	Guinea pig (circular layer)	0.89 ± 0.04	10.4 ± 0.1	$P < 0.001$
	Dog (longitudinal layer)	0.77 ± 0.10	10.5 ± 0.2	$P < 0.001$
	Dog (circular layer)	0.80 ± 0.05	9.2 ± 0.1	
Galanin-M35	Guinea pig (circular layer)	0.91 ± 0.07	9.3 ± 0.1	$P < 0.001$
	Dog (longitudinal layer)	0.75 ± 0.11	9.2 ± 0.2	$P < 0.001$
	Dog (circular layer)	0.78 ± 0.08	10.5 ± 0.2	

cells in which galanin induces a contraction (Table 4). On the contrary, in cells from dog ileum circular layer, calculation of pA2 values confirmed that M35 was more potent than M15 on the relaxing receptor type (Table 4). In both types of receptors, the slope of the Schild plots was nearly equal to 1, indicating that both antagonists were competitive. Indeed, in the experiments designed to build Schild plots, we obtained a maximal effect of galanin, either a contraction or a relaxation, as intense in the presence of antagonists as in their absence but at higher concentrations. In the longitudinal muscle layer of dog ileum, slopes observed for M15 and M35 were quite weak, although they were not statistically different from 1. The shape of the dose-response curves in this case was also in agreement with a competitive inhibition of the effect of galanin by M15 and M35. The small value of the slope observed in this layer could also suggest that galanin receptors on these cells could be heterogenous, the subtypes of which we could not distinguish with the pharmacological tools used in the present experiments.

In respect to the effects mediated, the observation of the pA2 values of M15 and M35 on the various muscle cells we studied indicates that the galanin receptor inducing a contraction and the receptor inducing a relaxation are pharmacologically and functionally distinguishable. Moreover, previous results from our laboratory indicate that galanin triggers different intracellular pathways in smooth muscle cells when it interacts with these two types of receptors. Indeed, in longitudinal muscle layer from dog colon[28] and circular muscle layer from pig ileum,[18] galanin induces a cell contraction via an activation of a pertussis toxin–sensitive G protein and an influx of extracellular calcium. In circular muscle layer from dog colon, galanin induces a cell relaxation by activating the adenylate cyclase.[28]

Galanin receptors are present on many tissues and cell types, mainly neuronal tissue[19] and pancreatic islet cells.[29] The antagonistic effect of M15 has been studied in the central nervous system[19] and pancreatic islet cells.[20] The comparison of the results of these previous studies with those of the present one shows that IC_{50} of M15 on galanin-induced cell contraction in guinea pig (80 pmol/L) and dog ileum (90 pmol/L) are in the same order as IC_{50} of M15 on the receptors of the central nervous system (100 pmol/L).[21] On the contrary, the IC_{50} of M15 on galanin-induced relaxation of dog intestinal circular muscle cells (900 pmol/L) is the same order as that of M15 observed on galanin-mediated inhibition of insulin release by pancreatic islet cells (1 nmol/L).[20] Thus, receptors for galanin on digestive smooth muscle could be divided into two subtypes, one of which seems to be similar to the receptor present at the central nervous system level and one to the receptor present on pancreatic islet cells. The distinction between these two types of receptors could later lead to a specific nomenclature, according to their predominant location.

Finally, galanin induces either a cell contraction or relaxation, depending on the species and the muscle layers studied, through the activation of different receptors, which are now pharmacologically distinguishable. To explain the various effects of galanin in vivo, the relevance of these distinct receptors may be taken into consideration.

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